AD_____

Award Number: DAMD17-03-1-0352

TITLE: Is Peripheral Benzodiazepine Receptor (PBR) Gene Expression Involved in Breast Cancer Suppression by Dietary Soybean Protein

PRINCIPAL INVESTIGATOR: Salil K. Das, Ph.D.

CONTRACTING ORGANIZATION: Meharry Medical College
Nashville, TN 37208

REPORT DATE: May 2005

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2005	Annual (1 May 04 -30 Apr 05)

4. TITLE AND SUBTITLE
Is Peripheral Benzodiazepine Receptor (PBR) Gene Expression Involved in Breast Cancer Suppression by Dietary Soybean Protein

5. FUNDING NUMBERS
DAMD17-03-1-0352

6. AUTHOR(S)
Salil K. Das, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Meharry Medical College
Nashville, TN 37208

E-Mail: sdas@mmc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Localization of PBR in nucleus, and nuclear cholesterol transport are implicated in breast cancer development. Our previous report suggested that the soybean protein may protect against the development of a more aggressive mammary gland adenocarcinoma. Furthermore, there was a delay in the development of breast cancer in the soybean group challenged with DMBA in comparison to the animals fed casein and challenged with DMBA. The objective of the present task was to confirm whether the beneficial effect of soybean protein in breast cancer development is mediated via PBRs. Breast cancer was developed by gavage administration of single dose of DMBA (80 mg/kg) into 50-day old female rats, maintained on a standard AIN-76A diet containing either 20% casein or 20% soybean protein. After 122 days of DMBA administration, the animals were sacrificed. All tumors were detected by palpation and at autopsy biopsy specimens were taken for histological grading. The casein group had 20% grade I (non-aggressive), 60% grade II (moderately aggressive) and 20% grade III (aggressive) mammary gland adenocarcinoma. However, the soybean group had 100% grade I adenocarcinoma and no aggressive grade II or grade III. Control animals had no tumor. B_{max} of PBRs was significantly higher in cancerous tissues than that in control tissues. However, the increase was significantly less in soybean protein group (53%) than that in casein group (128%). Nuclear cholesterol uptake as well as NTPase activity was significantly higher in cancerous tissues than control tissues. However, while there was no significant change in the cholesterol uptake between casein group and soybean protein group, endogenous cholesterol level was more in casein group than soybean group. These data suggest that PBR ligand binding, and PBR-mediated cholesterol transport into the nucleus and increase in NTPase may be involved in the aggressiveness of mammary gland adenocarcinoma, thus participating in the advancement of the disease.

14. SUBJECT TERMS
Breast Cancer Prevention, Soybean Protein, PBRs Gene Expression

15. NUMBER OF PAGES
13

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

The beneficial effects of dietary soybean protein in human health, particularly in breast cancer prevention, have been recently emphasized (1). However, to our knowledge, no information is available concerning the effects of dietary consumption of soybean protein on the expression of some genes, which may play a vital role in the prevention of breast cancer. It has recently been shown that ligand binding and mRNA expression of peripheral benzodiazepine receptor (PBR) is dramatically increased in the highly aggressive breast cancer cell lines and aggressive metastatic human breast tumor biopsies compared with nonaggressive cell lines and normal breast tissues (2). PBRs in aggressive breast cancer cell lines and tissue biopsies are mostly localized in and around the nucleus, which is in contrast to the largely cytoplasmic localization in nonaggressive cell lines and normal breast tissues. Furthermore, in aggressive cell lines, PBR drug ligands are found to increase the uptake of cholesterol by the nuclei and simultaneous incorporation of bromodeoxyuridine into the cells, suggesting the role of PBRs-mediated nuclear cholesterol uptake in cell proliferation (2). Numerous studies also implicate a role of nuclear cholesterol in the mechanisms underlying cell proliferation and cancer progression (2). It is not known whether the beneficial effect of dietary soybean protein on breast cancer suppression is mediated by its inhibitory effect on PBR expression, nuclear localization, and PBR-mediated cholesterol transport into the nucleus and cell proliferation. The objective of this project is to test the hypothesis that increased ligand binding, increased gene expression and possible mutation(s), and nuclear localization of PBRs, and PBRs-mediated cholesterol transport into the nucleus of breast epithelial cells are involved in cancer proliferation, and this aggressive phenotype expression can be prevented by dietary consumption of soybean protein.

WORK DONE DURING THE SECOND YEAR (May 1, 2004 – April 30, 2005)

APPROVED STATEMENT OF WORK

Task 1. To develop a breast cancer model by administration of DMBA to female rats fed a diet containing casein as the source of protein, and to inhibit the tumor development with soybean as the dietary protein (Months 1-18).

a. Feed weanling animals standard diets containing either casein or soybean protein and give DMBA by gavage and maintain the animals for 80 days.
b. Confirm breast cancer development in animals fed casein and suppression of breast cancer in animals fed soybean protein after 80 days of feeding.
c. Collect breast tissue for biochemical studies.
d. Develop and standardize methodologies for biochemical assays.

Task 2. To determine the role of PBRs in breast cancer suppression by dietary soybean protein (Months 18-36)

a. Maintain primary cultures of breast epithelial tissues.
b. Assay of PBR ligand binding in breast epithelial cells (Specific Aim 1).
c. Localize PBRs in cell nuclei by fluorescent microscopy (Specific Aim 2).
d. Measure nuclear uptake of cholesterol in breast epithelial cells (Specific Aim 3).
e. Measure bromodeoxyuridine uptake by breast epithelial cells (Specific Aim 4).
f. Measure ornithine decarboxylase activity of breast epithelial cells (Specific Aim 4).
g. Quantitate the expression c-fos in breast epithelial cells (Specific Aim 4).
h. Quantitate the expression of mRNA for PBRs in breast epithelial cells (Specific Aim 5).
i. Sequencing of the full-length cDNA for PBRs from breast epithelial cells (Specific Aim 6)

During this period we focused our studies primarily on completion of Task 1 and initiation of Task 2 as follows:

Development of Breast Tumor in Rats

Design of Animal Study

The first objective of this study was to produce breast cancer model in female rats, maintained on casein containing diet, by gavage administration of DMBA, and to investigate whether the tumor development can be counteracted by replacement of casein with soybean as dietary source of protein. So far we have carried out two separate experiments. For each experiment, 20 female Sprague Dawley rats were obtained at 21 days of age. They were divided into 4 groups. Animals from groups 1 and 2 received standard AIN-76A diet containing 20% casein and those of groups 3 and 4 received same diet containing 20% soybean protein instead of 20% casein. Diets were prepared by Harlan Teklad, WI. The diets were designed to be similar in nutrient content. The composition of diets was given in the Year 1 annual report.

Animals received food and water ad libitum. Animals of groups 2 and 4 received DMBA dissolved in sesame oil by gavage (15 mg per animal). Control animals (groups 1 and 3) received the vehicle only by gavage. Animals were weighed and also palpated twice weekly to detect breast tumors beginning four weeks after the administration of carcinogen. At the end of the study (postinjection time of 122 days), the animals were killed by carbon dioxide asphyxiation. All tumors were detected by palpation and at autopsy biopsy specimens were taken for histological analysis. Breast tissues were removed, quickly frozen in liquid nitrogen and stored at –80°C for direct biochemical analysis.

RESULTS:

Change in Body Weight. DMBA injection had no significant effects on the growth of animals. Furthermore, the growth pattern was similar in animals fed either casein or soybean protein. Data were shown in the Year 1 annual report

Time Course for Tumor Formation. Even though multiple tumors were observed in some animals of both groups, the number of tumors per rat was less in soyprotein group than casein group at each postinjection time. Data were presented in the Year 1 annual report.

Breast Tumor Incidence. Incidence of tumors was less in soybean protein group than that in casein group. Data were presented in Year 1 annual report.

Tumor Characteristics. There was no tumor in any animal which did not receive the carcinogen regardless of whether they were fed casein or soy protein. Even though there was a difference in the time course of tumor development between the casein group and the soyprotein group, the tumors were visibly apparent externally for both groups. Some tumors in both groups had darker area, possibly implicating cessation of angiogenesis in that area. However, the degree of discoloration was higher in animals fed soyprotein than that in animals fed casein. It is important that we investigate whether soyprotein retards the progression of angiogenesis. Furthermore, while the size of the largest tumor was not remarkably different between soyprotein group (3.5mm x 3.3mm x 1.2mm) and the casein group (3.8mm x 2.78mm x 1.6mm), the weight of the largest tumor was 44.5% lower in soybean group (7.52 g) than that in the casein group (13.54 g). It will be of interest to find out whether the tumors will shrink if the animals are fed soyprotein diet for longer period of time. Control animals had visible blood vessels. However, in soybean group, there was a dilation of blood vessels which may stimulate angiogenesis. Data were presented in Year 1 annual report.

Pathology of Breast Tumors. 100% of the mammary gland adenocarcinoma found in the soybean group was of the non-aggressive type (grade I). However, in the casein group, there was a higher percentage of aggressive tumors (20% grade I, 60% grade II, and 20% Grade III). Representative photographs of light microscopy from grade I tumor from the soybean group and grade II and grade III tumor from the casein group were shown in the Year 1 annual report.

Binding of ^{3}[H] Ro5-4864 to PBR in mammary gland. Differential ligand binding was observed in mammary gland. [^{3}H] Ro5-4864 bound specifically to PBR. PBR binding was characterized by Scatchard analysis. The observed k_d values of breast tissue suggest an increase of affinity for the receptor in DMBA-induced breast tumors (Table 1). B_{max} was significantly higher in DMBA-induced breast tumors than that in normal breast. However, dietary soybean protein caused significantly lesser increase (1.6-fold) in B_{max} than casein (2.3-fold).

Table 1. Binding Characteristics of PBRs in Normal and DMBA-Induced Rat Breast Tumors: Effects of dietary protein

Parameters	Casein	Casein + DMBA	Soybean	Soybean +DMBA
B_{max} (pmol/mg)	1.94 ± 0.54	4.43 ± 0.75*	1.8 ± 0.54	2.96 ± 0.99*
K_d (nM)	42.90 ± 17.1	5.93 ± 2.19	14.25 ± 0.55	2.29 ± 0.57

Cholesterol transport. Transport of cholesterol was determined as the incorporation of [^{3}H] cholesterol in intact nucleus acquired from normal and DMBA-induced breast tumors. Experiments were carried out using 10μM [^{3}H] cholesterol. Significant increase of cholesterol transport was observed in tumors compared to normal (Figure 1). No significant difference was noticed between casein-fed tumors and soybean-fed tumors.

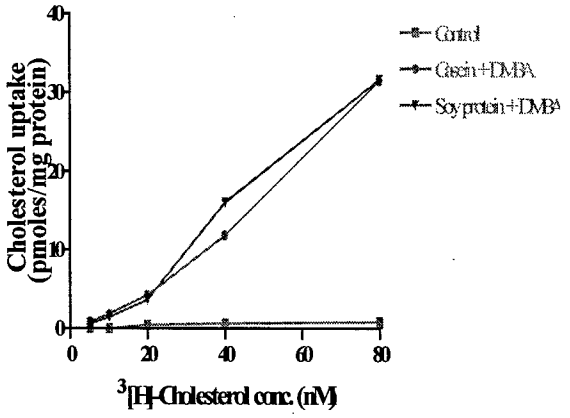


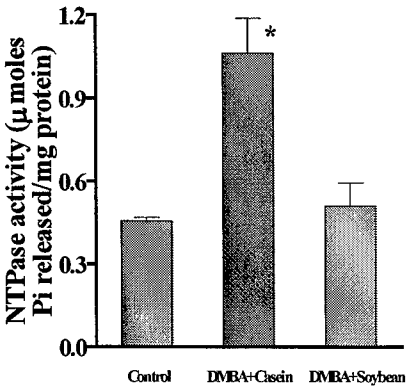
Figure 1

Binding of ^{3}[H] Ro5-4864 to nucleus PBR in mammary gland. The observed k_d values suggest an increase of affinity for the nuclear receptor in tumors (Table 2). B_{max} was remarkably higher in tumors than normal tissues. However, B_{max} was lower in soybean protein-fed tumors than casein-fed tumors.

Table 2. Binding Characteristics of PBRs in Nucleus of Normal and DMBA-Induced Rat Breast Tumors: Effects of Dietary Protein

Parameters	Control	Casein + DMBA	Soybean + DMBA
B_{max} (pmol/mg)	0.645 ± 0.045	44.87 ± 6.738*	21.70 ± 3.406*
K_d (nM)	8.91 ± 7.81	5.562 ± 1.309	6.828 ± 1.887

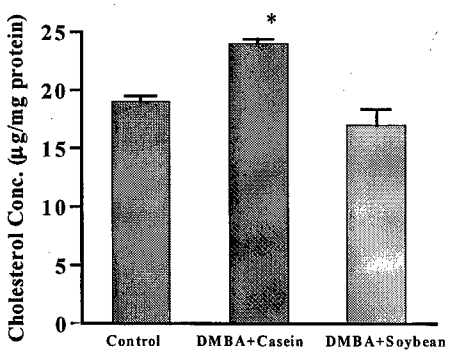
NTPase activity. According to Figure 2, NTPase activity was higher (130%) in breast tumors in comparison to normal mammary gland. NTPase activity in soybean protein group was at the same level as the control.



p<0.05

Figure 2

Endogenous nuclear cholesterol level. 26% increase in cholesterol level was observed in casein-fed tumors compared to normal gland (Figure 3). There was no significant change in cholesterol level in soybean protein-fed tumors compared to normal mammary gland.



*p<0.05

Figure 3

Western blot analysis. At the level of protein regulation, we measured changes of the 18-kDa and 32-kDa subunits of PBR separately (Figure 4). We found significant changes in the levels of both subunits when comparing the normal vs tumor tissues. In case of 32-kDa subunits, PBR expression was increased by 42.9% in casein-fed tumor than normal mammary gland (Figure 4A&B), whereas between control and soybean protein-fed tumor tissue no significant difference was observed. The expression of 18-kDa subunit of PBR was increased (45.5%) in casein-fed tumors in comparison to the normal (Figure 4C&D). Contrary to the 32-kDa subunit, a significant decrease (31.82%) of the expression of 18-kDa subunit was observed in soybean protein -fed tumors in comparison to the control animal.

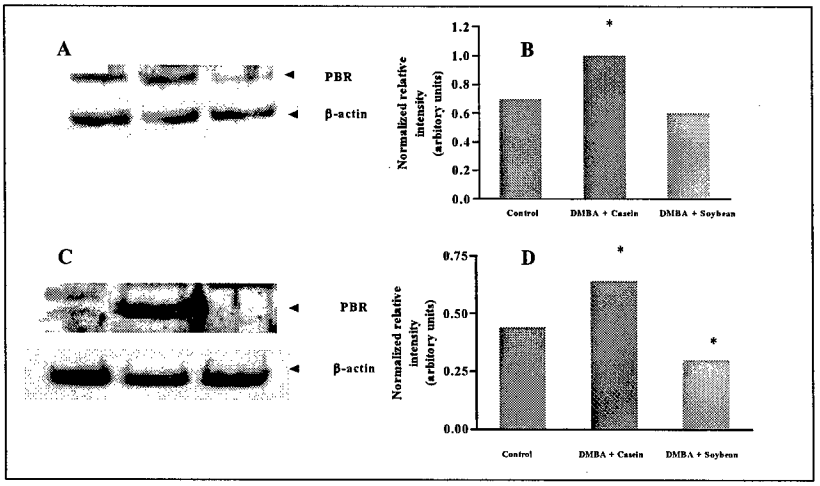


Figure 4

DISCUSSION

PBR and its endogenous ligand DBI have been detected in many benign and malignant tissues of various species. PBR and DBI have previously been detected in acinar cells of rat breast tissue and at a higher density, in DMBA-induced breast tumors [3]. In our experiments, we also find an increase in the number of receptors available for binding (B_{max}) (Table 1). In fact, tumor tissue shows lower k_d indicating higher affinity of ligand to these tissue. Based on these data the involvement of PBR and DBI in the regulation of function and growth of rat mammary cells may be suggested.

The physiological role of PBR is still debated. Its major function in endocrine tissues and some cell lines seem to be associated with cholesterol transport and steroidogenesis [4]. We have found an increase in the density of PBR in nucleus of breast tumors (Table 2), which may be responsible for increased transport of cholesterol into the nuclei of breast cancer tissue (both casein and soybean protein group) in comparison to normal tissue (Figure 1). Cholesterol is a

lipid found in many biological membranes. Studies have also implicated a role of nuclear cholesterol in mechanisms underlying cell proliferation and cancer progression [3,5]. We suggest that endogenous PBR ligands bind to PBR found on the nuclear membrane and facilitate cholesterol transport into the nucleus (Figure 1, Table 2). Cholesterol is then mobilized into the nucleus. Cholesterol's presence in the nucleus may change the dynamics of the nuclear membrane such as fluidity or associate itself as part of the nuclear membrane. When membrane fluidity is altered, signals that direct cell proliferation pathways indicate numerous signaling cascades in the cell [5].

Our results clearly demonstrate that the nuclear NTPase is sensitive to the cholesterol content of the nuclear membrane (Figures 2 & 3). Czubryt et al [6] showed that the nuclear membrane cholesterol increased in vivo and the NTPase activity increased with it. The incorporation of cholesterol into the nuclear membrane in the present study may alter NTPase activity via a change in membrane rigidity. The response of cholesterol-enriched nuclei suggests that cholesterol incorporation has left the membrane integrity more susceptible to damage from stressful stimuli like cancer. It is interesting to note that NTPase activity and endogenous cholesterol level in nuclei was lower in soybean group than casein group. This may be associated with the beneficial effect of dietary consumption of soybean protein in delaying the breast cancer progression.

The expression of 32-kDa subunit of PBR protein increases only in casein-fed tumors and not in soybean protein-fed tumors (Figure 4). Furthermore, 18-kDa subunit of PBR also increases in casein group, whereas, it is decreased in soybean group (Figure 4). Even though the exact mechanism is not known at this time, it may be suggested that 32-kDa proteins may be a polymorphic form of 18-kDa PBR proteins. It is known that aggressive human breast cancer cells contain mainly a PBR dimer, which increases cholesterol transport into the nucleus and cell proliferation [2]. Delavoie et al [7] also proposed that PBR polymer might be the functional unit responsible for ligand-activated cholesterol binding and that PBR polymerization is a dynamic process modulating the function of this receptor in cholesterol transport and other cell-specific PBR-mediated functions.

PBR nuclear localization and increase in cholesterol transport in breast cancer implicates that PBR has a role in nuclear functions. Many molecular and cellular changes are currently used as a factor in diagnosing breast cancers as prognostic indicators. Effective anticancer therapies are key in treating breast cancer. This study clearly indicates that PBR is an important molecule in cancer diagnosis and progression. Data on this study will provide a better understanding of the interplay involving PBR and other molecules especially cholesterol in the breast cancer signaling cascade. Furthermore, Soybean protein appears to have the beneficial effect in breast cancer development by down-regulating the expression of PBRs as well as nuclear cholesterol uptake.

Key Research Accomplishment:

1. Development of breast cancer model in rats by dietary feeding of casein as source of protein and administration of DMBA by gavage. Majority of cancers was of aggressive type (60% grade II and 20% grade III). Only 20% was of the grade I (non-aggressive) type.
2. Dietary consumption of soybean protein has a beneficial effect. It delays the onset of cancers and also produces a less aggressive cancer (100% non-aggressive). Therefore, prognosis will be better if soybean protein is consumed in lieu of casein as dietary source of protein.
3. Even though both casein and soybean fed animals had breast cancer when gavaged with DMBA, soybean protein consumption possibly retards the progression of Angiogenesis.
4. We have standardized the methods of isolation and culturing of rat mammary epithelial cells.
5. We have standardized all techniques in relation to the binding assay for peripheral Benzodiazepine receptors.
6. We have standardized the methods of nuclear cholesterol transport assay.
7. We have established that beneficial effect of soybean protein in delaying the progression of breast cancer is mediated by its down-regulation of the expression of PBRs.

Reportable Outcomes:

Manuscript:

1. Sinha Roy, S., Mukherjee, S., Mukhopadhyay, S., and Das, S. K. Differential Effect of Cadmium on Cholinephosphotransferase Activity in Normal and Cancerous Mammary Epithelial Cell Lines, Mol. Cancer Therapeutics 3(2): 199-204, 2004.
2. Mukhopadhyay, S., Das, S. K., and Mukherjee, S. Expression of Mn-Superoxide Dismutase Gene in Normal and Cancerous Human Mammary Epithelial Cells. Journal of Biomedicine and Biotechnology 2004: 4 (2004) 195-202.
3. Mukhopadhyay, S., Mukherjee, S. and Das, S.K. Increased Expression of Peripheral Benzodiazepine Receptor (PBR) in Dimethylbenz[a]anthracene-Induced Mammary Tumors in Rats. Submitted to Glycoconjugate J., April, 2005

Abstract:

1 Akech, J. and Das, S. K. Correlation between Expression of the Peripheral Benzodiazepine Receptors and Breast Cancer Cell Proliferation, FASEB J. 2004, 18(8), C109, IUBMB/ASBMB 2004 Meeting, Boston, MA, June 12-16, 2004.

2 Mukhopadhyay S, Ballard BR, Mukherjee S, Das SK. Potential Role of Peripheral Benzodiazepine Receptor (PBR) Expression as a Biomarker for Breast Cancer. 7th

International Symposium on Biochemical Roles of Eukarytic Cell Surface Macromolecules, Jan 10-14, 2005, Puri, India.

3 Ferguson M, Das SK and Mukherjee S. Role of Glyoxalase I and Glyoxalase II in Non-Tumorigenic and Tumorigenic Human Breast Call Lines. FASEB Meeting, April 2-6, 2005, San Diego, Ca.

4 SinhaRoy S, Mukhopadhyay S, Mukherjee S and Das SK. Modulation of Expression of Cholinephosphotransferase in Breast Cancer. FASEB Meeting, April 2-6, San Diego, CA.

5 Mukhopadhyay S, Ballard BR, Mukherjee S, and Das SK. Increased Expression of Peripheral Benzodiazepine Receptor (PBR) in Dimethylbenz[a]anthracene-Induced Mammary Tumors in Rats. FASEB Meeting, April 2-6, San Diego, CA.

6 Mukhopadhyay S, Ballard BR, Mukherjee S, Das SK. Increased Expression of Peripheral Benzodiazepine Receptor (PBR) in Breast Cancer. ERA of Hope 2005 DOD Breast Cancer Research Program Meeting, Philadelphia, PA, June 8-11, 2005.

7 Mukhopadhyay S, Mukherjee S, and Das SK. Beneficial Effects of Dietary Soybean Protein in Prevention of DMBA- Induced Breast Cancer in Rats. XVIII International Congress of Nutrition, Durban, South Africa, Sept 19-23, 2005.

CONCLUSIUONS:

Current results support the idea that soybean protein has a beneficial effect in controlling the aggressiveness in breast cancer progression. While, 100% of the breast tumor induced in rats fed soybean protein was of grade I (non-aggressive) type, casein consumption produced more aggressive tumors (20% grade I, 60% grade II and 20% grade III). The beneficial effect of soybean protein is mediated via down-regulation of the expression of the PBRs and nuclear cholesterol uptake.

REFERENCES:

1. Hakkak, R., Korourian, S., Shelnutt, S.R., Lensing, S., Ronis, M.J.J. and Badger, T. M. Diets containing whey proteins or soy protein isolate protect against 7,12-dimethylbenz@anthracene-induced mammary tumors in female rats, Cancer Epidemiology, Biomarkers & Prevention, Vol 9, 113-117, 2000.
2. Hardwick M, Fertikh D, Culty M, L, Li H, Vidic B and Papadopoulos V. Peripheral-type Benzodiazepine receptor (PBR) in human breast cancer: Correlation of breast cancer cell aggressive phenotype with PBR expression, nuclear localization, and PBR-mediated cell proliferation and nuclear transport of cholesterol. Cancer Research 59: 831-842, 1999.
3. Brown MS, Goldstein JL, The SREBP pathway: Regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor, *Cell* **89**, 331-340 (1997).
4. Papadopoulos V, Amri H, Boujrad N, Cascio C, Culty M, Garnier M, Hardwick M, Li H, Vidic B, Brown AS, Reversa JL, Bernassau, JM, Drieu, K, Peripheral benzodiazepine receptor in cholesterol transport and steroidogenesis, *Steroids* **62**, 21-28 (1997).

5. Singleton PA, Bourguignon LY, CD44 interaction with ankrin and IP_3 receptor in lipid rafts promotes hyaluran-mediated Ca^{2+} signaling leading to nitric oxide production and endothelial cell adhesion and proliferation, *Exp Cell Res* **295**, 102-118 (2004).
6. Czubryt MP, Ramjiawan B, Pierce GN, The nuclear membrane integrity assay, *Mol Cell Biochem* **172**, 97-102 (1997).
7. Delavoie F, Li H, Hardwick M, Robert JC, Giatzakis C, Peranzi G, Yao ZX, Maccario J, Lacapere JJ, Papadopoulos V, In vivo and in vitro peripheral-type benzodiazepine receptor polymerization: functional significance in drug ligand and cholesterol binding, *Biochemistry* **42**, 4506-4519 (2003).